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Association of maternal Toll-like receptor-4 alleles with susceptibility to early-onset preeclampsia in central Greece

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ABSTRACT

Introduction: Altered maternal inflammatory responses may play a role in the development of hypertensive disorders of pregnancy like preeclampsia, its more severe early-onset form and intrauterine growth restriction. We evaluated the relation of common allelic variants of Toll-like receptor 4 (TLR4), known to impair the inflammatory response, with the susceptibility to early-onset preeclampsia in Central Greece.

Methods: We compared the occurrence of TLR4 (Asp299Gly and Thr399Ile) alleles in heterozygous (A/G, C/T) and homozygous (G/G, T/T) states in 84 women with a history of early-onset preeclampsia and 94 age matched controls with a history of only uneventful pregnancies, by direct sequencing.

Results: Heterozygous TLR4 allelic variants were more common in women with a history of early-onset preeclampsia than in controls (GA for Asp299Gly: 14.3% vs 6.4% (AA), p = 0.053 & CT for Thr399Ile: 16.7% vs. 6.4% (CC), p = 0.019) and a stronger association was obtained when homozygous allelic carriers were also included (GA/GG for Asp299Gly: 16.7% vs. 6.4% (AA), p = 0.03 & TC/TT for Thr399Ile: 19.0% vs. 6.4% (CC), p = 0.01).

Discussion: We recorded association between common TLR4 gene variants and early-onset preeclampsia. Our findings support the involvement of maternal innate immune system in severe hypertensive disorders of pregnancy and point to the potential value of maternal TLR4 polymorphisms as predictors-risk factors of susceptibility to early-onset preeclampsia in Central Greece.

1. Introduction

Preeclampsia (PE) is a complex multi-system disease of placental origin and one of the leading causes of maternal and perinatal morbidity and mortality, affecting 3%–5% of all pregnancies [1,2]. PE is characterized by hypertension and the coexistence of one or more of the following: proteinuria, maternal organ dysfunction, which is defined by new onset of any of the following (Renal insufficiency, hepatic dysfunction, neurological complications - eclampsia, stroke, confusion, hyperreflexia, severe headache, blindness or persistent visual scotomata and hematological complications, disseminated intravascular coagulation or hemolysis and uteroplacental dysfunction (fetal growth restriction) [3–5]. It usually presents after 20 weeks' gestation, and, when of early-onset (defined as < 34 + 0 weeks), is associated with greater maternal and fetal risks, including intrauterine growth retardation, hypoxia and fetal loss [6,7]. Several risk factors have been used as predictors including family history of PE, PE in previous pregnancy, chronic hypertension, first pregnancy, new paternity, age and race (very young and older than 40 and black women have higher risk), obesity, multiple pregnancy, interval between pregnancies (less than two or more than 10 years apart), chronic migraines, diabetes type 1 or 2, kidney disease and IVF. Influenced by both genetic and environmental risk factors, the development of PE presents multi-factorial inheritance, indicating the potential contribution of parental genetic profile in its pathogenesis [8–12].

Maternal adaptation to pregnancy is essential for normal growth

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and development of semi-allogeneic fetus. It requires properly regulated innate and adaptive immune components to effectively control the inflammatory response to various threats. Inappropriate inflammatory patterns have been related to abnormal trophoblastic invasion [13] and endothelial damage [14] and implicated in the pathogenesis of common placental and hypertensive disorders of pregnancy, including pre-eclampsia, its more severe early onset form and intra-uterine growth restriction [3,14,15].

Infusion of low-dose endotoxin or lipopolysaccharide (LPS), a potent antigen normally present in gram-negative bacteria known to induce a systemic pro-inflammatory reaction, in rats, induced a PE-like syndrome, including hypertension, proteinuria and glomerular endotheliosis [16]. LPS-mediated inflammation releases pro-inflammatory cytokines through activation of pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), which are key elements of innate immunity. LPS recognition by TLR4 sensors leads to production of nuclear factor kappa B and release of pro-inflammatory cytokines (IL-1b, IL-6, TNF-a) that signal cells of the adaptive immune system (mainly T cells) to constitute an inflammatory response necessary for effective clearance of the harmful pathogen. Allelic variants of TLR4 have been associated with an attenuated immune response to LPS, as well as to exogenous and endogenous pathogenic insults, leading to inappropriate inflammatory patterns which can be both ineffective in dealing with an infectious agent, as well as cause the adverse effects of uncontrolled inflammation [17–21].

The human TLR4 (MIM C603030) gene, see also Diagram 1, is located on chromosome 9q33.1 and its polymorphic alleles Asp299Gly or D299G or (A896G) (MIM 603030.001) and Thr399Ile or T399I or (C1196T) (MIM 603030.002) known to influence the endotoxin TLR4-sensing system, have been related to sepsis [22], premature delivery [23], atherogenesis [24] and early-onset preeclampsia (EOP) albeit with conflicting results [4,7,25].

We assessed the susceptibility and relative risk of EOP in carriers of common TLR4 polymorphisms from Central Greece.

2. Materials and methods

2.1. Participants

Study participants (N = 178) were recruited in the tertiary referral center of Central Greece (Thessalia University Hospital). Inclusion criteria for cases (N = 84) were a history of EOP and delivery before 34 weeks of gestation and for healthy age matched women controls (N = 94), a history of one or more uneventful pregnancies. At inclusion, clinical and demographic data and whole blood samples for DNA isolation were obtained from all participants. The study was approved by the Medical Ethics Committee of Thessalia University Hospital, and written informed consent was obtained from all participants. EOP was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy for preeclampsia symptoms [5], which is: increased blood pressure (≥140 mmHg SBP or ≥90 mmHg DBP on two or more occasions at least 6 h apart) that occurred before 34 weeks of gestation in a woman with previously normal blood pressure, accompanied by proteinuria (≥0.3 g/24 h). Fetal growth restriction was diagnosed if the fetal birth weight was below the 10th percentile for gestational age [26]. Exclusion criteria were chronic hypertension, diabetes mellitus, autoimmune disease and renal disease. All study participants were Caucasians, inhabiting the area of central Greece (Thessalia). The clinical characteristics of study participants are summarized in Table 1.

Table 1
Clinical characteristics of study groups.

	Control N = 94 N (%)	EOP N = 84 N (%)	p
Parity			
1	68 (72.3)	62 (73.8)	0.826[¶]
2	14 (14.9)	10 (11.9)	
3	12 (12.8)	12 (14.3)	
Age (years), mean (SD)	31.8 (5.8)	31.8 (6.3)	0.941[+]
Fetal sex			
Boys	48 (51.1)	48 (57.1)	0.661[*]
Girls	42 (44.7)	32 (38.1)	
Boy-Girl (twins)	4 (4.3)	4 (4.8)	
Fetal loss			
No	94 (100)	80 (95.2)	0.048[*]
Yes	0 (0)	4 (4.8)	
Twins-pregnancy			
No	86 (91.5)	74 (88.1)	0.453[¶]
Yes	8 (8.5)	10 (11.9)	
Week of gestation, median (IQR)	39 (38–40)	32 (31–34)	< 0.001[++]
Preterm delinery			
No	92 (97.9)	0 (0)	< 0.001[¶]
Yes	2 (2.1)	84 (100)	
Birth weight (gr), median (IQR)	3550 (3170–3900)	1650 (1260–2140)	< 0.001[++]

[¶] chi-square test.
[*] Fisher's exact test.
[+] Student's *t*-test.
[++] Mann-Whitney test.

2.2. Genotypic analysis

DNA isolation. Genomic DNA was extracted from 200 µl peripheral blood using the QIAmp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany).

PCR amplification. A 438bp-long fragment containing both TLR4 Asp299Gly and Thr399Ile polymorphisms, see also Diagram 1, was amplified from genomic DNA as previously described. Specific primer

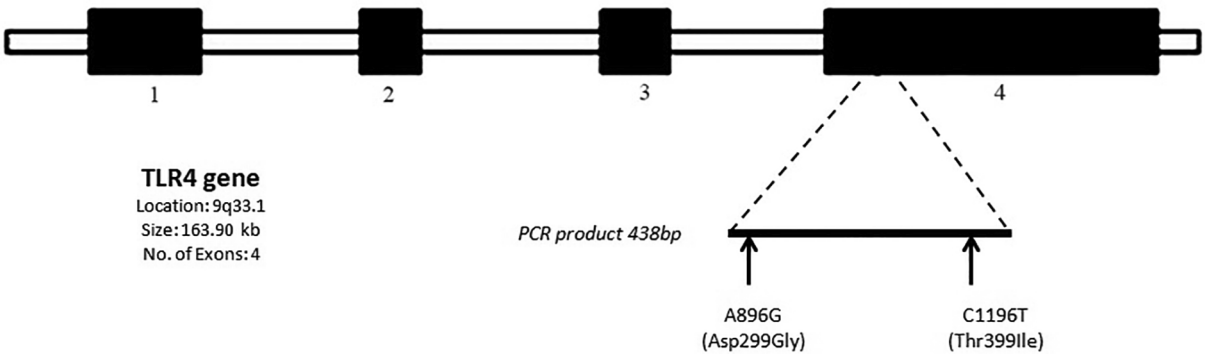

Diagram 1. TLR4 gene organization. Broken lines point the location of PCR product and arrows the location of the two common polymorphic alleles studied (adapted from Ref. [33]).

pair sequences were designed by Primer 3 software (www.justbio.com): forward 5′-TCTAGAGGGCCTGTGCAATT-3′ and reverse 5′-TGAAACTC ACTCATTTGTTTTCAA-3′. PCR reaction mixtures contained 20 mMTris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 1.5U Taq DNA polymerase (Invitrogen) and 15 pmol of each primer. Amplification products were generated following 35 cycles of DNA denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec in a PTC-200MJ Research Thermocycler (MJ Research, Inc., Waltham, MA, USA).

Sequence analysis. Purification of the PCR products was performed using the PureLink PCR purification kit (Invitrogen). Automated cycle sequencing was performed with both strands in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems) analyzer using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Sequences were aligned relative to control sequences from GenBank (GenBank accession number: NM 138554), using Sequencer PC software (Gene Codes Corp., Ann Arbor, MI, USA) and examined for the presence of mutations.

2.3. Statistical analysis

Normal distributed variables are expressed as mean and standard deviation (SD); while variables with skewed distribution are expressed as median and interquartile range (IQR). Qualitative variables were expressed as absolute and relative frequencies. The normality assumption was evaluated using Kolmogorov-Smirnov test. If the normality assumption was satisfied for the comparison of means between two groups, Student's *t*-test was used. Mann-Whitney test was used for the comparison of continuous variables between two groups when the distribution was not normal. For the comparisons of proportions chi-square tests and Fisher's exact tests were used. A logistic regression analysis was performed to associate genotypes with the likelihood of EOP. Unadjusted and adjusted odds ratios with 95% confidence intervals were computed from the results of the logistic regression analyses. Statistical significance was set at 0.05 and analyses were conducted using SPSS statistical software (version 22.0).

3. Results

Regarding the basic clinical characteristics between the two age-matched study groups, mean age, 31.8 years (SD = 5.8) for EOP and 31.8 years (SD = 6.3) for control, no statistical differences were detected in terms of parity, fetal sex and twin pregnancies. As expected, mean gestational week at delivery and birth weight were significantly lower ($p < 0.001$), while fetal death was elevated ($p < 0.048$) in EOP relative to control cases (Table 1).

The presence of “wild type” (AA) and (CC) and “mutant” Asp299Gly or D299G (hetero-GA & homozygous-GG) & Thr399Ile or T399I (hetero-TC & homozygous-TT) sequences at position 896 and 1196 of the TLR4 gene, was recorded in 178 age matched study participants, 84 with EOP and 94 without EOP (controls), by direct sequencing (Table 2). Heterozygous TLR4 allelic variants were more common in women with a history of EOP than in controls (GA for Asp299Gly: 14.3% vs 6.4% for AA, p = 0.053 & CT for Thr399Ile: 16.7% vs. 6.4% for CC, p = 0.019) and a stronger association was observed when homozygous allelic carriers were also included (GA/GG for Asp299Gly: 16.7% vs. 6.4% for AA, p = 0.03 & TC/TT for Thr399Ile: 19.0% vs. 6.4% for CC, p = 0.01). No statistically significant differences with pregnancy outcomes, like parity, fetal sex, fetal loss, twins, week of gestation and birth weight were recorded in women with EOP between wild type (AA & CC) and mutant (GA/GG & TC/TT) TLR 4 allelic carriers (Tables 3 and 4).

As expected by their close proximity (300bp), the two polymorphic alleles co-segregate (4, 28). We observed a total of 18 Asp299Gly and 20 Thr399Ile homo (GG/TT) & heterozygous (AG/CT) mutant alleles in the 20 polymorphic carriers of our study (Table 2). Unadjusted and adjusted for odds ratios from logistic regression analysis showed that women with GA/GG or TC/TT genotypes in amino acid receptor positions 299 and 399 respectively, had greater likelihood for EOP. Unadjusted odds ratios OR (95% CI) were 2.91 (1.07–8.03) for those with GA/GG and 3.45 (1.28–9.29) for those with TC/TT, as compared to their wild type controls AA and CC, respectively. When adjusted for mother's age, parity and twin pregnancies, the corresponding odds ratios were marginally improved to 2.94 (1.06–8.18) and 3.52 (1.28–9.60), respectively (Fig. 1).

Table 2
Distribution of TLR 4 genotypes in study groups.

		Group				P
		Control		EOP		
		N	%	N	%	
Asp299Gly	AA	88	93.6	70	83.3	0.053*
	GA	6	6.4	12	14.3	
	GG	0	0	2	2.4	
Thr399Ile	CC	88	93.6	68	81.0	0.019*
	TC	6	6.4	14	16.7	
	TT	0	0	2	2.4	
Asp299Gly	AA	88	93.6	70	83.3	0.030¶
	GA/GG	6	6.4	14	16.7	
Thr399Ile	CC	88	93.6	68	81.0	0.010¶
	TC/TT	6	6.4	16	19.0	

¶ chi-square test.
* Fisher's exact test.

Table 3
Association of Asp299Gly GA/GG with EOP pregnancy outcomes.

	TLR 4: Asp299Gly GA/GG		
	AA N (%)	GA/GG N (%)	p
Parity			
1	50 (71.4)	12 (85.7)	0.245¶
2	8 (11.4)	2 (14.3)	
3	12 (17.1)	0 (0)	
Fetal sex			
Boys	40 (57.1)	8 (57.1)	> 0.999¶
Girls	26 (37.1)	6 (42.9)	
Boys-Girl (twins)	4 (5.7)	0 (0)	
Fetal loss			
No	68 (97.1)	12 (85.7)	0.128¶
Yes	2 (2.9)	2 (14.3)	
Twins			
No	62 (88.6)	12 (85.7)	0.670¶
Yes	8 (11.4)	2 (14.3)	
Week of gestation, median (IQR)	32 (31–34)	32 (27–34)	0.750+
Birth weight (gr), median (IQR)	1655 (1260–2140)	1420 (850–2600)	0.675+

¶ Fisher's exact test.
+ Mann-Whitney test.

4. Discussion

The relative distribution of mutant TLR4 Asp299Gly and Thr399Ile alleles was assessed in women with and without EOP in a Caucasian population from central Greece. In agreement with previous reports (4, 6), our findings suggest that carriers of these mutant alleles are more susceptible to EOP. These mutant alleles alter extracellular PRR motifs, causing inefficient immune response to diverse common exogenous and endogenous pathogenic insults in early stages of placental implantation and later development up to 20 weeks of gestation [27]. To understand the biological significance of our findings, the natural history of mutant TLR4 alleles must be taken into account.

Table 4
Association of Thr399Ile TC/TT with EOP pregnancy outcomes.

	TLR 4: Thr399Ile TC/TT		
	CC N (%)	TC/TT N (%)	p
Parity			
1	48 (70.6)	14 (87.5)	0.221¶
2	8 (11.8)	2 (12.5)	
3	12 (17.6)	0 (0)	
Fetal sex			
Boys	38 (55.9)	10 (62.5)	0.905¶
Girls	26 (38.2)	6 (37.5)	
Boys-Girl (twins)	4 (5.9)	0 (0)	
Fetal loss			
No	66 (97.1)	14 (87.5)	0.162¶
Yes	2 (2.9)	2 (12.5)	
Twins			
No	60 (88.2)	14 (87.5)	> 0.999¶
Yes	8 (11.8)	2 (12.5)	
Week of gestation, median (IQR)	32 (31–34)	32.5 (29–34)	0.727+
Birth weight (gr), median (IQR)	1650 (1260–2115)	1560 (1075–2480)	0.833+

¶ chi-square test.
+ Mann-Whitney test.

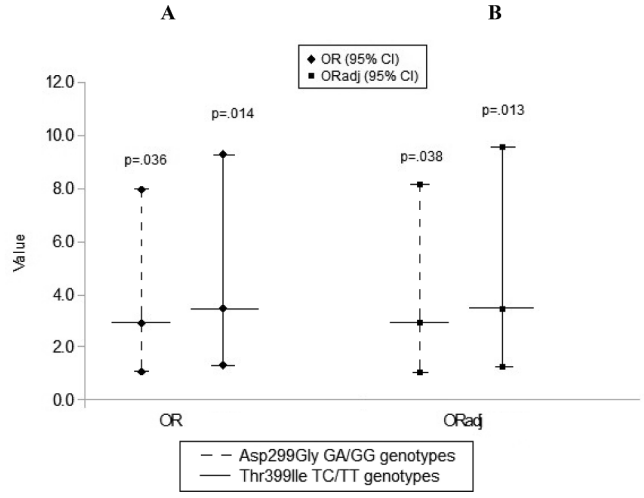

Fig. 1. Results from logistic regression analysis for the association of: A. Asp299Gly GA/GG genotypes with EOP: Unadjusted and adjusted for odds ratios from logistic regression analysis showed that women with GA/GG genotype in amino acid receptor position 299, had greater likelihood for EOP. Unadjusted odds ratio OR (95% CI) was 2.91 (1.07–8.03) for those with GA/GG, as compared to their wild type controls AA. When adjusted for mother's age, parity and twin pregnancies, the corresponding odds ratio was marginally improved to 2.94 (1.06–8.18). B. BThr399Ile TC/TT genotypes with EOP: Unadjusted and adjusted for odds ratios from logistic regression analysis showed that women with TC/TT genotype in amino acid receptor positions 399, had greater likelihood for EOP. Unadjusted odds ratio OR (95% CI) was 3.45 (1.28–9.29) for those with TC/TT, as compared to their wild type controls CC. When adjusted for mother's age, parity and twin pregnancies, the corresponding odds ratio was marginally improved to 3.52 (1.28–9.60).

Mutant Asp299Gly TLR4 allele originated first in Africa to offer protection from malaria associated mortality, counterweighing the increased susceptibility to infections and septic shock [19,20]. Later addition of Thr399Ile mutant allele in Indo-Europeans reduced the risk for septic shock to rates similar to wild type allelic carriers [19,20] and was a useful addition as Asp299Gly alone, would had caused a bigger threat to major plaque, typhoid fever and influenza outbreaks in Europe.

Until its extinction in late 1950s, malaria was endemic in central Greece, site of origin of our study participants, resulting in the fixation of high frequencies of sickle trait and mutant TLR4 alleles in locals relative to other native Greek populations not exposed to malaria threat [28,29]. Recent relief of selective pressures from infections by aggressive antimicrobial practices in central Greece is expected to negate the role of malaria protective alleles, like Asp299Gly leading to eventual reduction in their frequencies. While the forces of population genetics complemented by active carrier screening and genetic counseling reduced the frequency of sickle trait to less than half in central Greece over the last fifty years [29], the anticipated reduction of Asp299Gly frequency, mainly by genetic drift, is expected to follow a much longer path. The absence of Asp299Gly in two of the 20 mutant carriers of our study may mark the beginning of such declining trend in central Greece.

On these grounds, we may speculate on the relative risk of mutant TLR4 haplotypes to develop EOP in response to pathogenic threats. First we predict that the double GG/TT mutant *Asp299Gly/Thr399Ile* allele known to alter receptor binding to ligands, reduce functional TLR4 levels and mount blunt immune response would confer the highest degree of susceptibility to EOP following TLR4 sensed pathogenic insults [30]. Next in descending susceptibility should follow double GA/TC *Asp299Gly/Thr399Ile* and single TC *Thr399Ile* heterozygotes. The tight association of these polymophic alleles with EOP (Tables 3 and 4), along with its minor influence by common confounders (Fig. 1), supports their pivotal role in the development of pathogenic insult-triggered hypertensive disorders of pregnancy. Further analyses with greater numbers of EOP cases from central Greece will be required to validate our predictions. Consolidation of these trends and assignment of relative risk to various TLR4 polymorphisms may justify their potential incorporation and refine the local panel of predictive EOP guidelines.

Within the limits of our relatively small sample size, we observed association between mutant TLR4 alleles and pathogenic insult-triggered EOP, in a population from central Greece. Given that EOP is a complex hypertensive disorder of pregnancy of unknown etiology, with negative impact on maternal and fetal morbidity and mortality [1–3] as well as on off-springs of EOP pregnancies [31,32], our findings may aid to improve its prevention. The performance of available guidelines for EOP prediction, is likely to be enhanced by screening populations with relatively high mutant carrier frequencies for EOP-associated polymorphisms, like those for TLR4 in central Greece [28,29], shown to confer an over 3 fold elevated risk for EOP to local carriers (Fig. 1).

In conclusion, our promising pilot findings support provisional inclusion of prognostic TLR4 Asp299Gly and Thre399Ile screening to local EOP guidelines for improved management and prevention of major hypertensive disorders of pregnancy in central Greece.

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Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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